Lung Adenocarcinoma with EGFR Amplification Has Distinct Clinicopathologic and Molecular Features in Never-Smokers

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Abstract

In a subset of lung adenocarcinomas, the epidermal growth factor receptor (EGFR) is activated by kinase domain mutations and/or gene amplification, but the interaction between the two types of abnormalities is complex and unclear. For this study, we selected 99 consecutive never-smoking women of East Asian origin with lung adenocarcinomas that were characterized by histologic subtype. We analyzed EGFR mutations by PCR-capillary sequencing, EGFR copy number abnormalities by fluorescence and chromogenic in situ hybridization and quantitative PCR, and EGFR expression by immunohistochemistry with both specific antibodies against exon 19 deletion–mutated EGFR and total EGFR. We compared molecular and clinicopathologic features with disease-free survival. Lung adenocarcinomas with EGFR amplification had significantly more EGFR exon 19 deletion mutations than adenocarcinomas with disomy, and low and high polysomy (100% versus 54%, \( P = 0.009 \)). EGFR amplification occurred invariably on the mutated and not the wild-type allele (median mutated/wild-type ratios 14.0 versus 0.33, \( P = 0.003 \)), was associated with solid histology (\( P = 0.008 \)), and advanced clinical stage (\( P = 0.009 \)). EGFR amplification was focally distributed in lung cancer specimens, mostly in regions with solid histology. Patients with EGFR amplification had a significantly worse outcome in univariate analysis (median disease-free survival, 16 versus 31 months, \( P = 0.01 \)) and when adjusted for stage (\( P = 0.027 \)). Lung adenocarcinomas with EGFR amplification have a unique association with exon 19 deletion mutations and show distinct clinicopathologic features associated with a significantly worsened prognosis. In these cases, EGFR amplification is heterogeneously distributed, mostly in areas with a solid histology. [Cancer Res 2009;69(21):OF1–8]

Introduction

A subset of non–small cell lung carcinomas (NSCLC) has activating mutations in the epidermal growth factor receptor (EGFR) that predict response to treatment with EGFR tyrosine kinase inhibitors (TKI; refs. 1–3). The highest prevalence of somatic EGFR mutations (up to 55%) is in never-smoking women of East Asian origin (4–6). In contrast, somatic K-RAS mutations occur more commonly in patients with a smoking history and seem to be exclusive of EGFR mutations (7, 8).

EGFR mutations mostly occur in exons 18 to 21 and cluster in two major hotspots (1). The most common mutations, L858R substitution mutation in exon 21 (40% of EGFR mutations) and small in-frame deletions in exon 19 (>50%), are reported to be the most closely associated with radiographic responses with EGFR TKI therapy (9, 10). Mutations in exons 18 and 20 account for 10% of EGFR mutations in NSCLC (1).

The EGFR gene is also one of a growing number of putative oncogenes that can undergo marked copy number changes (11). The significance of EGFR copy number changes in NSCLC has not been clearly established, but several clinical trials have shown that a higher number of EGFR copies is associated with a better response to treatment with gefitinib (12–15).

EGFR mutant tumors and NSCLC cell lines sometimes have concurrent increases in EGFR copy number (8, 16–19). Activating kinase domain mutations seem to be an early event in lung carcinogenesis (20, 21), and several studies have hypothesized that increased EGFR copy number is associated with more aggressive and poorly differentiated lung carcinomas (18, 22, 23).

Interestingly, studies in human NSCLC cell lines have shown differential EGFR and downstream signaling activation based on the type of mutation and the presence of gene amplification (24). These findings, corroborated with clinical observations of improved survival in EGFR TKI–treated patients with exon 19 deletion mutations (9, 25), suggest that certain molecular abnormalities are associated with distinctive clinicopathologic characteristics. In this study, we explored kinase domain mutations and copy number abnormalities in lung adenocarcinomas from a cohort of patients known to have a high prevalence of EGFR mutations and examined their pathologic and clinical significance. We found that a subset of patients with lung adenocarcinomas have a distinct genetic profile, clinicopathologic characteristics, and outcome.

Materials and Methods

Patients Selection

Surgical pathology specimens were obtained from 99 consecutive never-smoking women with NSCLC treated initially by surgery at the Kaohsiung Veterans General Hospital, Taiwan from 1999 to 2004. Of these, 65 cases were included in the study based on a pathologic diagnosis of lung adenocarcinoma, absence of neoadjuvant treatment, and the availability

References


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of sufficient tissue to examine molecular abnormalities, gene copy number alterations, and protein expression by immunohistochemistry. H&E staining was performed on 5-μm sections from paraffin-embedded, formalin-fixed tumor tissue. Adenocarcinomas were evaluated by two pathologists (L.R. Chirieac and L.M. Sholl) and classified according to WHO criteria (26). In mixed subtype adenocarcinomas, we assessed the percentage of each histologic pattern (acinar, papillary, solid, and bronchioloalveolar) in 10% increments and recorded the most predominant histologic pattern (27). The study was approved by the Institutional Review Board of the Kaohsiung Veterans General Hospital and Kaohsiung Medical University, and all participants gave written informed consent.

**Analysis of the EGFR and KRAS Mutations**

DNA was extracted from areas of paraffin slides containing >50% tumor and mutation analysis for EGFR exons 18 to 21 and KRAS exons 12 to 13 was performed using SURVEYOR (Transgenomic) digestion and/or RFLP analysis followed by high-performance liquid chromatography on the Transgenomic WAVE Nucleic Acid High Sensitivity Fragment Analysis System (WAVE HS system, Transgenomic) according to previously published protocols (28). Amplicons were fractioned to enrich for heteroduplexes, and collected fractions were amplified and sequenced. EGFR-mutated and wild-type alleles were quantified using quantitative PCR (DxS EGFR mutation test kit, DxS, Ltd.) in cases with EGFR exon 19 deletion E746_E750 (n = 29) and L858R mutation (n = 10) for which sufficient DNA remained following mutation analysis.

**Analysis of EGFR Copy Number**

**Fluorescence in situ hybridization.** Fluorescence in situ hybridization (FISH) was performed on paraffin-embedded tissue sections according to protocols described previously (29). At least 50 cells were evaluated for each case by two pathologists (L.M. Sholl and A.J. Iafrate). The EGFR gene copy number was classified according to previously published criteria (14, 29) as disomy, low polysomy (54 copies of EGFR in >40% of cells), high polysomy (54 copies of EGFR in >40% of cells), or gene amplification (homogenously staining regions with ≥15 copies in ≥10% of cells) (or a gene/chromosome ratio per cell of ≥2).

**Chromogenic in situ hybridization.** Chromogenic in situ hybridization (CISH) was performed on paraffin-embedded tissue sections and scored according to protocols described previously (29) using the EGFR amplification probe (Invitrogen/Zymed).

**Immunohistochemistry Analysis for EGFR Overexpression**

Immunohistochemistry for total EGFR protein was performed using Dako (Dako North America, Inc.) clone H11 at a 1:100 dilution following a 10-min proteinase digestion step. EGFR expression was classified according to previously published criteria into one of four categories: 0 for no or only cytoplasmic staining; 1+ for partial membrane staining; 2+ for weak, complete membrane staining in >10% of tumor cells; and 3+ for intense, complete membrane staining in >10% of tumor cells (30, 31). Expression scores of 2 or 3 were defined as EGFR protein overexpression.

**Statistical Analysis**

Fisher's exact test and Wilcoxon rank-sum test were used to analyze the association of EGFR copy number changes with each of the molecular, pathologic, and clinical characteristics, respectively, for categorical and continuous data. Disease-free survival was measured from the date of surgery to the date of NSCLC recurrence or death, whichever occurred earlier, and was censored for patients still alive without a recurrence at their last follow-up. In order to adjust for the underlying prognosis due to differential stage, the log rank test was stratified by stage in the comparison of disease-free survival. The data analysis was conducted primarily using SAS 9.1 (SAS Institute), whereas StatXact 6.1 (Cytel Software, Corp.) was used to perform statistical analysis.

**Figure 1.** EGFR mutation status, copy number abnormalities, and EGFR protein expression in lung adenocarcinoma and relationship with the predominant histology. Each row is a lung cancer sample. Black rectangles, the results of EGFR sequencing (EGFR sequence) or a predominant pattern (Predominant histology). EGFR expression was categorized on a scale from 0 (absent staining) to 3 (strong staining) and illustrated on a gray scale from white (score = 0), light gray (score = 1), dark gray (score = 2), and black (score = 3). BAC, bronchioloalveolar histology.
compute an exact $P$ value for the stratified log rank test. All $P$ values are based on a two-sided hypothesis.

**Results**

**EGFR Mutation Status and EGFR Copy Number Abnormalities**

The results of EGFR molecular and copy number analyses are summarized in Fig. 1 and illustrated in Fig. 2A. As expected, this subset of patients (2, 3, 7) had a high prevalence of EGFR mutations in the studied adenocarcinomas. Some adenocarcinomas have a concomitant mutation in the EGFR gene (dotted columns). In the EGFR-amplified cancers, there is a significant difference in the mutation frequency of EGFR exon 19 deletions ($P = 0.009$) but not in other mutations. B, furthermore, in cancers with EGFR exon 19 deletions, there is an increase in both wild-type and mutated EGFR copies in low and high polysomy cases (mean deleted/wild-type ratios, 1.14 and 1.21, respectively), whereas there is a preferential increase of the deleted allelic in amplified cases (mean deleted/wild-type ratios, 36.3). The Y-axis indicates the log10 ratio between deleted allele and the wild-type allele.

Figure 2. Comparison of the genetic alterations and pathologic characteristics among the lung adenocarcinomas according to the EGFR copy number categories. Lung adenocarcinomas with EGFR amplification have very distinct genetic profiles. A, prevalence of EGFR mutations in the studied adenocarcinomas. Some adenocarcinomas have a concomitant mutation in the EGFR gene (dotted columns). In the EGFR-amplified cancers, there is a significant difference in the mutation frequency of EGFR exon 19 deletions ($P = 0.009$) but not in other mutations. B, furthermore, in cancers with EGFR exon 19 deletions, there is an increase in both wild-type and mutated EGFR copies in low and high polysomy cases (mean deleted/wild-type ratios, 1.14 and 1.21, respectively), whereas there is a preferential increase of the deleted allelic in amplified cases (mean deleted/wild-type ratios, 36.3). The Y-axis indicates the log10 ratio between deleted allele and the wild-type allele. C, proportion of solid histology in each carcinoma. Columns, mean; bars, SE. D, EGFR protein expression.

A significant increase in the ratio of exon 19 deletion mutation EGFR allele to wild-type allele was detected only in the adenocarcinomas with EGFR amplification (median value, 14; range, 2–162) when compared with adenocarcinomas with disomy, low polysomy, and high polysomy (median, 0.3; range <0.1 to 7.0, $P = 0.003$; Fig. 2B). The mutant alleles could not be detected by quantitative PCR in 2 of the 29 cases (one case with EGFR reported (8). One case had both the L858R activating mutation and T790M resistance mutation (Fig. 1).

The exon 19 deletion mutation was present in 47%, 63%, and 52% of adenocarcinomas with disomy, low polysomy, and high polysomy, respectively (overall average in nonamplified cases, 54%), and in 100% of adenocarcinomas with EGFR amplification (Fisher’s exact test, $P = 0.009$; Table 2 and Fig. 2A). An L858R mutation was also detected in an amplified case, but only in the presence of a coexisting exon 19 deletion mutation (Figs. 1 and 2D).

A significant increase in the ratio of exon 19 deletion mutation EGFR allele to wild-type allele was detected only in the adenocarcinomas with EGFR amplification (median value, 14; range, 2–162) when compared with adenocarcinomas with disomy, low polysomy, and high polysomy (median, 0.3; range <0.1 to 7.0, $P = 0.003$; Fig. 2B). The mutant alleles could not be detected by quantitative PCR in 2 of the 29 cases (one case with EGFR
amplification and one case with high polysomy) with exon 19 deletion mutation that were tested. We did not identify any case in which the L858R-mutated allele was amplified (ratio of mutant to wild-type allele, ≤1.0).

Lung Carcinomas with EGFR Amplification Have Distinctive Clinical and Pathologic Characteristics

Clinical features. The clinical features of patients with lung adenocarcinomas with and without EGFR gene amplification are presented in Table 2. Patients with adenocarcinomas with EGFR amplification presented predominantly in advanced clinical stages: stage IIA (two patients), stage IIIA (five patients), and stage IV (two patients). None of the patients with adenocarcinomas with EGFR amplification presented with stage I, whereas 52% of all other patients presented with stage I (P = 0.009). There were no significant differences between the stages at presentation of patients with adenocarcinomas with exon 19 deletion and those with L858R (data not shown). We found no significant differences in the types of adjuvant treatment between patients with or without EGFR amplification (P = 0.508; Table 2).

Histologic features. A predominant solid pattern was present in 5 of 9 (56%) adenocarcinomas with EGFR amplification in contrast to only 7 of 56 (13%) adenocarcinomas without amplification (P = 0.008; Table 2; Fig. 2C). Predominant acinar and papillary patterns were present in similar proportions in adenocarcinomas from all other copy number categories and a BAC predominant pattern occurred most commonly in disomic cases (Table 2). Although solid histology was more frequently seen in adenocarcinomas with exon 19 deletion mutation than in those with the L858R mutation (20% versus 9%), this difference was not significant (P = 0.66).

Immunophenotype. EGFR protein overexpression was significantly more prevalent in adenocarcinomas with EGFR amplification than adenocarcinomas with any other copy number alterations (Fig. 2D). EGFR protein was overexpressed in 8 of 9 (89%) adenocarcinomas with EGFR amplification, 7 of 23 (30%) with high polysomy, 3 of 16 (19%) with low polysomy, and 1 of 17 (6%) with disomy (P < 0.001; Table 2; Fig. 2D). Thirteen of 14 (93%) adenocarcinomas with exon 19 deletion mutation identified by sequencing were positive by immunohistochemistry using the exon 19 deletion–specific antibody (32). Similar to the total EGFR antibody, the deletion mutation–specific antibody had increased expression in the tumors with EGFR gene amplification (Fig. 3).

Overexpression of EGFR with exon 19 deletion was heterogeneously distributed in EGFR gene–amplified lung cancer specimens, mostly in areas with a solid growth pattern.

Survival

Median follow-up time was 27 months. The disease-free survival rate was significantly better for patients who had pathologic stage I carcinoma than it was for patients with more advanced stages (42.8% versus 17.5% at 5 years, P = 0.047). The median disease-free survival time was significantly worse among patients with adenocarcinoma with EGFR amplification than it was among patients without amplification (16 versus 31 months, P = 0.01; Fig. 4). When adjusted for stage, patients with EGFR amplification had a significantly worse outcome (P = 0.027).

EGFR Copy Number Is Heterogeneous within Individual Tumors

We used CISH, an alternative technique to FISH, in order to visually locate the regions with EGFR copy number abnormalities in the tumor samples. As we previously reported, EGFR copy number abnormalities assessed by CISH highly correlate with FISH (29). Of the adenocarcinomas with EGFR amplification by FISH, five specimens had a heterogeneous copy number distribution in different histologic patterns when examined by CISH (Fig. 3). By CISH, the areas with BAC and acinar histology had low polysomy and areas with solid and papillary histology had EGFR amplification. EGFR protein expression using both the total EGFR antibody and the exon 19 deletion–specific antibody was most consistently and strongly detected in the tumor cells with EGFR gene amplification. Absent or low EGFR protein expression (scores 0 and 1) were seen in the regions with BAC and acinar histology, in areas of low polysomy.

Discussion

In this study, we show that in this selected cohort of patients with lung adenocarcinomas, EGFR amplification (a) is invariably associated with exon 19 deletion mutations, (b) occurs exclusively...
on the mutated allele, (c) is focally distributed mostly in regions with solid histology, and (d) is associated with worse outcome.

Our study has several unique features. First, the demographic characteristics of the study population (never-smoking women of East Asian origin) favor the presence of somatic EGFR mutations, and we identified EGFR kinase domain mutations in 80% of the cases. As expected in this patient group, KRAS mutations were rare events. The homogeneity of the study population enabled us to identify highly significant associations between genetic, clinical, and pathologic features in the absence of a large population analysis. Second, we studied surgical resection specimens from patients who had no preoperative neoadjuvant treatment. Because lung tumors are heterogeneous, the availability of large tumor specimens eliminates the artifacts of sampling error. This is particularly problematic in assessing copy number abnormalities, which as our study reveals, could vary even within a single microscopic field of tumor. Finally, using a novel exon 19 deletion-specific antibody recently described by our group (32), we were able to directly visualize the location of tumor cells with EGFR exon 19 deletion mutations and show heterogeneity in receptor overexpression among different tumor cells. These findings might have implications for the efficacy of TKI treatment in any given patient with an EGFR-mutant adenocarcinoma. According to previously published criteria for classifying lung adenocarcinomas based on EGFR copy number abnormalities (13), 49% of the cases in our study would be considered "FISH positive" (EGFR high polysomy or gene amplification; ref. 13). The importance of distinguishing between high polysomy and gene amplification is not well-established; however, in our study, tumors containing EGFR with high polysomy did not have significantly unique clinical or pathologic features and were therefore grouped with all other non–gene-amplified tumors for statistical analyses. We found that gene amplification occurred exclusively in the tumors with EGFR exon 19 deletion mutations and did not occur in the tumors with wild-type EGFR or other types of EGFR mutations (Fig. 2 A).

### Table 2. Clinical characteristics of patients with lung adenocarcinoma according to EGFR copy number abnormalities

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<th>Characteristic</th>
<th>All patients (N = 65)</th>
<th>Disomy (N = 17)</th>
<th>Low polysomy (N = 16)</th>
<th>High polysomy (N = 23)</th>
<th>Gene amplified (N = 9)</th>
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<td>62 (46–78)</td>
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<td>66 (45–70)</td>
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<td>7 (50)</td>
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NOTE: Due to rounding not all percentages total 100.

*P value was based on the comparison between patients with EGFR amplification and all other patients.

†Stage II to IV only.
we found that EGFR amplification occurs preferentially on the mutated allele, an observation that strongly supports the concept that kinase domain mutations are early events in lung adenocarcinoma tumorigenesis, whereas gene amplification occurs later (Fig. 2B). In our study, the increase in the ratio of mutated/wild-type alleles occurred exclusively in cases containing the exon 19 deletion mutation.

The exclusive association of exon 19 deletion mutations with gene amplification is a novel observation and contrasts with prior studies that have identified gene amplification in different mutational backgrounds. Gene amplification has been detected in cell lines harboring either exon 19 deletion mutations or L858R mutation (17, 24). Previous studies have identified EGFR amplification in other genetic backgrounds, including in tumors that are EGFR wild-type and that harbor other EGFR mutations (15). However, in contrast with our study of untreated tumors, these combined alterations were identified in patients who had received prior chemotherapy (15).

Figure 3. Heterogeneous histology, EGFR copy number, and protein expression in a gene-amplified lung adenocarcinoma. Top, tumor field containing acinar and solid subtypes of adenocarcinoma. Bottom, H&E stain (magnification, ×400), total EGFR and EGFR exon 19 deletion protein expression by immunohistochemistry (IHC, magnification, ×400), and EGFR CISH (magnification, ×600). A, acinar histology with low polysomy by CISH and 1+ protein expression. B, solid histology with gene amplification in the form of homogenous staining regions by CISH and 3+ protein expression.
Recent published studies in selected NSCLC cell lines suggest mechanistic differences between tumors containing the exon 19 deletion mutation and those with the L858R mutation (24). Tumor cells containing the exon 19 deletion mutation show constitutive EGFR autophosphorylation only in the presence of gene amplification, whereas cells containing the L858R mutation show constitutive autophosphorylation regardless of amplification status (24). These findings, in association with evidence that amplification occurs only on the mutated EGFR allele (24, 33), suggest that amplification provides a growth advantage for tumor cells harboring the exon 19 deletion mutation.

Our analysis provides interesting clinical and pathologic correlations that validate the in vitro studies of EGFR receptor biology (24). We found that this subset of EGFR-amplified tumors was significantly more likely to contain high-grade, solid-subtype adenocarcinoma (Fig. 2C; ref. 26). Interestingly, this tumor subtype has been previously associated with KRAS mutations and a history of smoking (27); however, analysis of histologic subtypes in an EGFR mutation–enriched patient population has not been performed to date. We also found that EGFR-amplified tumors are more likely to present at higher stages and have worse disease-free survival (Fig. 4).

In this population of previously untreated lung adenocarcinomas, EGFR gene amplification is predicated on the existence of an underlying exon 19 deletion mutation, suggesting that, in at least a subset of patients, there is a stereotypical tumor progression pathway, with kinase domain mutations occurring early and gene amplification occurring later. Previous reports have shown that EGFR mutations are detectable even in preinvasive lesions such as atypical adenomatous hyperplasia, but increased EGFR copy number are mostly present in invasive (22) and higher grade tumors (23). Using an antibody directed specifically at exon 19 deletion–mutated EGFR, we showed that all histologic subtypes of the sequencing-proven mutant tumors contain at least low levels of mutant protein expression. Using CISH to evaluate copy number changes by light microscopy, we showed that gene amplification is heterogeneous, occurring preferentially in solid and high-grade papillary components. The tumor areas containing gene amplification highly correlated with the areas of protein overexpression by immunohistochemistry whether detected using the total EGFR antibody or exon 19 deletion–specific EGFR antibody. The fact that EGFR molecular alterations are heterogeneous distributed within lung adenocarcinoma specimens confirms previous reports (18, 23), and suggests that thorough pathologic and molecular analyses of a resection specimen are required to provide the most accurate information regarding tumor biology. Because the studied population was restricted to East Asian patients, the applicability of these findings to Western patients remains unclear.

Our findings show that within a subset of EGFR-mutated lung adenocarcinomas, EGFR gene amplification is associated with tumor progression, reflected by higher histologic grade and clinical stage. Amplification seems to promote constitutive receptor activation in exon 19 deletion–mutated NSCLC cell lines, and as such, may also provide a growth advantage for these cells (24). These findings highlight the complexity of tumor morphogenesis and suggest that molecular classification of lung cancer by both mutation and copy number analyses may have important implications for selection of therapy. Our study provides support for the concept of tumor heterogeneity and suggests a mechanism for variable responses to directed therapy for lung adenocarcinoma.

Disclosure of Potential Conflicts of Interest

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